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Hormonal regulation of phospholipase D activity in Ca^{2+} transporting cells of rabbit connecting tubule and cortical collecting duct

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Abstract

Phospholipase D (PLD) is distributed widely in mammalian tissues where it is believed to play an important role in the regulation of cell functions and cell fate by a variety of extracellular signals. In this study, we used primary cultures of rabbit connecting tubule (CNT) and cortical collecting duct (CCD) cells, grown to confluence on a permeable support, to investigate the possible involvement of PLD in the mechanism of action of hormones that regulate Ca^{2+} reabsorption. RT-PCR revealed the presence of transcripts of PLD1b and PLD2, but not PLD1a, in these cultures. Moreover, the expression of substantial amounts of PLD1 protein was demonstrated by Western blotting. To measure PLD activity, cells were labelled with [^{3}H]myristic acid after which the PLD-catalysed formation of radiolabelled phosphatidylethanol ([^{3}H]PtdEth) was measured in the presence of 1% (v/v) ethanol. Deamino-Cys,D-Arg^8-vasopressin (dDAVP) and N^6-cyclopentyladenosine (CPA), two potent stimulators of Ca^{2+} transport across these monolayers, stimulated PLD activity as was indicated by a marked increase in [^{3}H]PtdEth. Similarly, ATP, a potent inhibitor of dDAVP- and CPA-stimulated Ca^{2+} transport, increased the formation of [^{3}H]PtdEth. PLD activity was furthermore increased by 8Br-cAMP and following acute (30 min) stimulation of protein kinase C (PKC) with a phorbol ester (PMA). Chronic PMA treatment (120 h) to downregulate phorbol ester-sensitive PKC isoforms did not affect PLD activation by dDAVP, CPA and 8Br-cAMP, while markedly decreasing the effect of ATP and abolishing the effect of PMA. The PKC inhibitor chelerythrine significantly reduced PLD activation by dDAVP, CPA and 8Br-cAMP, without changing the effect of ATP. The inhibitor only partially reduced the effect of PMA. This study shows that Ca^{2+} transporting cells of CNT and CCD contain a regulated PLD activity. The physiological relevance of this activity, which is not involved in Ca^{2+} reabsorption, remains to be established.

Keywords: Cortical collecting duct; Connecting tubule; Ca^{2+} reabsorption; Vasopressin; ATP; N^6-Cyclopentyladenosine; Chelerythrine; Phorbol ester

1. Introduction

In mammals, the distal part of the nephron is the major regulatory site for Ca^{2+} excretion in the urine [1,2]. Hormone-regulated active Ca^{2+} reabsorption proceeds against a transepithelial electrochemical

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gradient and involves several components, each of which forms a potential target of hormone action. In the first place, a Ca^{2+} influx pathway, probably the recently cloned epithelial Ca^{2+} channel (ECaC) [3], present in the luminal membrane of the cell allows Ca^{2+} to passively flow into the cytosolic compartment down a steep electrochemical gradient. Next, a vitamin D-dependent Ca^{2+} binding protein (calbindin-D_{28K}) binds Ca^{2+} with high affinity and shuttles it to the basolateral membrane. Finally, a plasma membrane Ca^{2+}-ATPase and Na^{+}–Ca^{2+} exchanger transport Ca^{2+} out of the cytosol against a steep electrochemical gradient. Presently, it is unknown which component of the Ca^{2+} reabsorptive mechanism is subject to hormone-activated second messenger systems.

Previous work has shown that Ca^{2+} reabsorption in primary cultures of cells from rabbit connecting tubule (CNT) and cortical collecting duct (CCD) is stimulated by various hormones, including parathyroid hormone (PTH) [4,5], vasopressin [4–6], adenosine [4,7], prostaglandin E_2 [4,5] and atrial natriuretic peptide [8], and that ATP, which in itself does not affect basal Ca^{2+} reabsorption, significantly inhibits the effect of the stimulatory hormones [9,10]. It was demonstrated that the action of the stimulatory hormones was independent of cAMP, insensitive to chronic phorbol ester treatment and markedly inhibited by the protein kinase C (PKC) inhibitor chelerythrine, suggesting the involvement of a phorbol ester-insensitive, chelerythrine-inhibitable PKC isoform [4]. By contrast, chronic phorbol ester treatment was shown to abolish the inhibitory effects of both the phorbol ester and ATP on hormone-stimulated Ca^{2+} reabsorption [8,10,11]. These findings were taken as evidence that ATP acts primarily via a downregulatable PKC isoform to inhibit hormone-stimulated Ca^{2+} reabsorption at the level of the pathway involving the non-downregulatable PKC isoform [9]. The possibility of a non-cAMP-mediated mechanism involved in hormone-stimulated Ca^{2+} reabsorption was first considered by Poujeol and coworkers studying calcitonin-induced Ca^{2+} mobilisation in rabbit convoluted distal tubule cultures [12]. A major role of PKC in PTH-stimulated Ca^{2+} reabsorption was also deduced from studies with immortalised mouse distal convoluted tubule cells and primary cultures of mixed mouse CNT and cortical thick ascending limb cells [13].

The finding that differential activation of phorbol ester-sensitive and -insensitive PKC isoforms is the underlying principle in the action of inhibitory and stimulatory hormones on Ca^{2+} reabsorption raises the question of the mechanism of activation of these isoforms. The physiological activator of PKC is diacylglycerol and the hormone-induced formation of this lipid involves the action of hormone-sensitive phospholipases such as phosphatidylinositol-specific phospholipase C (PtdIns-PLC) and phosphatidylcholine-specific phospholipase D (PtdCho-PLD) [14,15]. The latter phospholipase hydrolyses PtdCho to yield phosphatidic acid and it has been proposed that phosphatidic acid is rapidly converted to diacylglycerol to activate PKC [16]. Since the diacylglycerols derived from PtdIns and PtdCho differ in fatty acid composition it is very well possible that they may activate different PKC isoforms [17]. In a recent study, evidence was provided that PTH acts through a pathway other than that involving PtdIns-PLC to promote the formation of PKC activating diacylglycerols [18]. PTH was shown to activate PtdCho-PLD and it was suggested that PTH acts through this pathway to increase diacylglycerol. Hormone-induced increases in PLD activity were also reported in rabbit CCD cells [19]. In this latter study, bradykinin-induced PLD activation was shown to be paralleled by inhibition of sphingomyelinase activity thus excluding the possibility that bradykinin acts through ceramide to activate PLD.

It has been demonstrated that agonists which stimulate the hydrolysis of PtdCho also cause the formation of phosphatidyl alcohols as a result of the PLD-catalysed transphosphatidylation reaction [20]. The present study uses this PLD-specific transphosphatidylation reaction to test the possibility that PLD is selectively activated by either the inhibitory or the stimulatory hormones of Ca^{2+} transport.

2. Materials and methods

2.1. Materials

Collagenase A and hyaluronidase were obtained

from Boehringer (Mannheim, Germany) and deamino-Cys,D-Arg^8-vasopressin (dDAVP) from Bachem Feinchemikalien (Bubendorf, Switzerland). N^6-Cyclopentyladenosine (CPA), ATP, 8Br-cAMP, indomethacin, 12-*O*-tetradecanoyl phorbol 13-acetate (PMA) and 2,2,4-trimethylpentane were purchased from Sigma (St. Louis, MO, USA). Chelerythrine and pertussis toxin were obtained from Research Biochemicals International (Cologne, Germany) and [9,10(*n*)-^{3}H]myristic acid (53 Ci/mmol) from The Radiochemical Centre (Amersham, UK). HPTLC silica-gel 60 plates, ethyl acetate and chloroform were purchased from Merck (Darmstadt, Germany), phospholipid standards from Avanti Polar Lipids (Birmingham, AL, USA), and 2′,5′-dideoxyadenosine (DDA) from Biomol (Plymouth Meeting, PA, USA). All other chemicals were of reagent grade.

2.2. Primary cultures of rabbit kidney cortical collecting system

Rabbit kidney CNT and CCD cells were immunodissected from kidney cortex of young New Zealand White rabbits (~0.5 kg) with antibody R2G9 and set in primary culture on permeable supports (0.33 cm^2; Costar, Cambridge, MA, USA) as described in detail previously [21]. The culture medium was a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DME/F12; Gibco, Paisley, UK) supplemented with 5% (v/v) decomplemented foetal calf serum, 50 μg/ml gentamicin, 10 μl/ml non-essential amino acids (Gibco), 5 μg/ml insulin, 5 μg/ml transferrin, 50 nM hydrocortisone, 70 ng/ml prostaglandin E_1, 50 nM Na_2SeO_3, and 5 pM triiodothyronine, equilibrated with 5% CO_2–95% air at 37°C. PLD measurements were performed 8 days after seeding the cells.

2.3. RNA extraction and reverse transcription (RT)-PCR

Total RNA was isolated from rat brain and primary cultures of CNT and CCD cells using Trizol (Gibco, Paisley, UK) and reverse-transcribed with M-MLV reverse transcriptase using random hexamer-mixed oligonucleotides. Specific primers for the amplification of PLD1 transcripts were designed on the basis of published sequences from rat liver [22]. Primers were chosen such that they made it possible to discriminate between PLD1a and PLD1b. The sense and antisense primers were 5′-GCCTATGGAAGGTGGGACGAC-3′ and 5′-GGAGTACCTGTCAATGAAATCAGC-3′, respectively. Primers to specifically amplify PLD2 transcripts were designed on the basis of the published sequence of rat brain PLD2 [23]. In this case, the sense and antisense primers were 5′-GAACAGGGGCAGTGTTTCCGA-3′ and 5′-CGCTGTTTCTTGCCACAGCTG-3′, respectively. The PCR of PLD1 and PLD2 transcripts was based on 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with an elongation time of 10 min at 72°C following the last cycle. DNA products, visualised by means of ethidium bromide staining, were scanned with an Imaging Densitometer (Bio-Rad, Munich, Germany).

2.4. Western blotting

Total cell lysates (100 μg) from primary cultures of rabbit CNT and CCD cells were subjected to SDS–PAGE. Proteins were transferred overnight to polyvinylidine difluoride membranes (Immobilon P, Millipore, Bedford, MA, USA). Membranes were blocked for 1 h at room temperature with phosphate-buffered saline (PBS) (pH 7.4) containing 1% (w/v) milk powder and 0.1% (v/v) Tween-20 (PBST-MLK). Subsequently, the blots were incubated overnight at 4°C with the rabbit anti-hPLD1 antibody described by Müller-Wieprecht et al. [24] diluted 1:1000 in PBST-MLK. The antibody was kindly provided by Prof. Dr. C.C. Geilen (University Medical Center Benjamin Franklin, The Free University of Berlin, Germany). Immunoreactive protein was detected by incubating the blots for 1 h at room temperature with goat anti-rabbit IgG antibody conjugated to alkaline phosphatase diluted 1:1000 in PBST-MLK. Membranes were washed with PBS (pH 7.4) containing 0.1% (v/v) Tween-20 and stained with 0.1 M diethanolamine, 0.34 mg/ml nitroblue tetrazolium, 0.18 mg/ml 5-bromo-4-chloro-3-indolylphosphate and 1.0 M $MgCl_2$. The membranes were scanned with an Imaging Densitometer (Bio-Rad, Munich, Germany).

2.5. PLD measurements

PLD measurements were performed essentially as described previously [25,26]. Briefly, monolayers, labelled with [^{3}H]myristic acid (3 μCi/ml) for 24 h, were washed and incubated in DME/F12 containing 5 μM indomethacin for another 3 h. Subsequently, the monolayers were washed and preincubated in a physiological salt solution (PSS) containing 140 mM NaCl, 2 mM KCl, 1 mM K_2HPO_4, 1 mM KH_2PO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM L-alanine, 5 μM indomethacin and 10 mM HEPES (adjusted to pH 7.40 with Tris) for 15 min. Next, the monolayers were washed and incubated in PSS containing 1% (v/v) ethanol and the agonist(s) of interest for another 30 min. At 30 min, the filter was excised and rapidly transferred into a lipid tube (Sarstedt, Essen, Germany) containing 1 ml of methanol, 500 μl of chloroform and 400 μl of water (4°C). The extract was mixed vigorously and phase separation was achieved by addition of 400 μl of chloroform and 400 μl of water followed by centrifugation. The lower organic phase was collected and blown to dryness with N_2. The dried extracts were dissolved in 20 μl of chloroform/methanol (9:1, v/v) and the lipids were separated by TLC using potassium oxalate-treated silica-gel 60 HPTLC plates. The plates were developed with the upper phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10, v/v). Radioactive spots corresponding to authentic lipid standards were visualised by I_2 staining and scraped off. Radioactivity was measured by liquid scintillation spectrometry.

2.6. Analysis of the data

The data presented are expressed as the mean ± S.E.M. of the number of experiments indicated. Overall statistical significance was determined by analysis of variance. In the case of significance ($P < 0.05$), individual groups were compared according to Fisher. P values of less than 0.05 were considered significant.

3. Results

To investigate the PLD isoform composition of primary cultures of rabbit CNT and CCD cells we performed RT-PCR using specific primers for PLD1, which allowed us to discriminate between the two splice variants of PLD1, PLD1a and PLD1b, and PLD2. Fig. 1A shows the three PLD transcripts in total RNA from rat brain for comparison. Cultured rabbit CNT and CCD cells clearly expressed PLD1b and PLD2. In contrast, the cells did not express transcripts of PLD1a. Western blot analysis of total cell lysate was performed to examine the expression of PLD1b at the protein level. Using an antibody directed against the C- and N-terminal sequences of human PLD1 we observed a positive band of expected size of ~110 kDa (Fig. 1B). These data show that primary cultures of rabbit CNT and

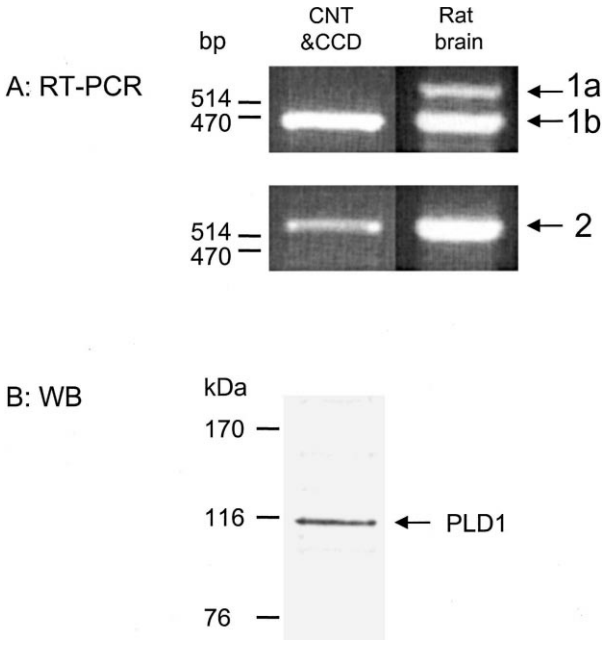

Fig. 1. Identification of PLD transcripts and PLD1 protein in primary cultures of rabbit CNT and CCD cells. (A) Specific primers for PLD1, which allowed us to discriminate between the PLD1 slice variants, PLD1a and PLD1b, and PLD2 were used to amplify cDNA fragments. In rat brain, the PLD1-specific primers yielded a larger product of 550 bp and a smaller product of 450 bp, representing PLD1a and PLD1b, respectively (upper panel; left lane), and one product with the PLD2-specific primers (lower panel; left lane). Primary cultures of rabbit CNT and CCD cells expressed the PLD1b and PLD2 fragments (right lanes). (B) Total cell lysates from primary cultures of rabbit CNT and CCD cells were subjected to SDS–PAGE and subsequently transferred to polyvinylidine difluoride membranes by Western blotting. The membranes were probed with a specific anti-human PLD1 antibody. The figure shows an immunoreactive band with an apparent mass of 110 kDa.

CCD express significant amounts of PLD1b at both the mRNA and the protein level.

In order to assess the effects of various hormones and second messenger systems involved in the regulation of Ca^{2+} reabsorption on PLD activity, [^{3}H]myristic acid-labelled monolayers of rabbit CNT and CCD cells were stimulated in the presence of 1% (v/v) ethanol for 30 min after which the amount of radiolabelled phosphatidylethanol ([^{3}H]PtdEth) was determined. Incubations were performed in the presence of indomethacin (5 μM) added 3 h prior to stimulation to inhibit the production and release of autostimulatory prostanoids [5]. Under these conditions, basal [^{3}H]PtdEth formation amounted to $0.44 \pm 0.03\%$ ($n = 10$) of total [^{3}H]myristic acid-labelled lipid. To study the role of PKC in hormone-induced PLD activation, monolayers were pretreated with PMA (0.1 μM) for 120 h. This treatment is widely used to selectively downregulate phorbol ester-sensitive PKC isoforms. Chronic PMA treatment slightly increased basal [^{3}H]PtdEth formation to $0.58 \pm 0.02\%$ ($n = 9$) of total labelled lipid. The data presented hereafter are corrected for basal [^{3}H]PtdEth formation.

Both the phorbol ester PMA (0.1 μM; both sides; 30 min) and the membrane-permeable cAMP analogue 8Br-cAMP (0.1 mM; both sides; 30 min) markedly increased the formation of [^{3}H]PtdEth (Fig. 2). The amount of [^{3}H]PtdEth formed during this acute treatment with PMA was 2.9-fold higher than that obtained with 8Br-cAMP. Since both drugs were added at high concentrations, this indicates that the PKC pathway is much more effective than the protein kinase A (PKA) pathway in increasing PLD activity. The combination of PMA and 8Br-cAMP did not further increase the amount of [^{3}H]PtdEth, suggesting the absence of a synergistic interaction between the two pathways. Chronic PMA treatment (120 h) virtually abolished the stimulatory effect of acute PMA treatment, while leaving that of 8Br-cAMP intact.

ATP (0.1 mM; both sides) stimulated the formation of [^{3}H]PtdEth to the same extent as 8Br-cAMP (Fig. 3A). The increase obtained with the combination of ATP and 8Br-cAMP was approximately 1.8-fold higher than that obtained with each of the two stimuli alone. The stimulatory effect of ATP was more than halved following chronic PMA treatment

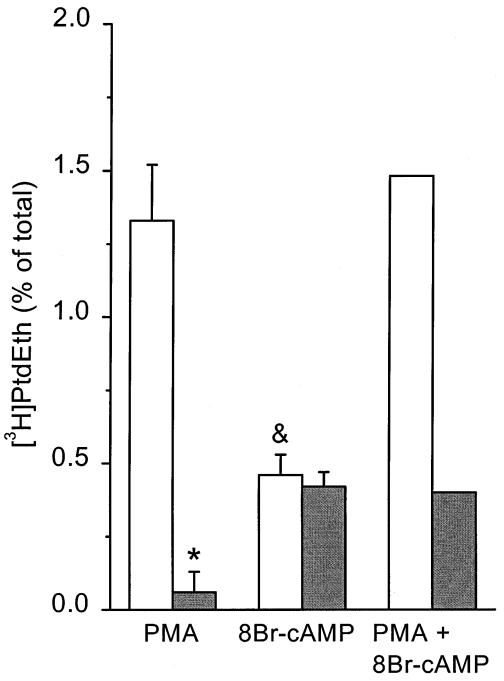

Fig. 2. Chronic phorbol ester treatment does not affect 8Br-cAMP activation of PLD but abolishes the increase in PLD activity following acute phorbol ester activation of PKC. [^{3}H]Myristic acid-labelled primary cultures of rabbit CNT and CCD cells were incubated with the indicated stimulants in the presence of 1% (v/v) ethanol for 30 min. Additions were: the phorbol ester PMA (0.1 μM; both sides), and the membrane-permeable cAMP analogue 8Br-cAMP (0.1 mM; both sides). To downregulate phorbol ester-sensitive PKC isoforms, monolayers were incubated in the presence of PMA (0.1 μM; both sides) for 120 h (closed bars). For each filter, the amount of [^{3}H]PtdEth is expressed as the percentage of total labelled lipid that was extracted. The data presented for PMA and 8Br-cAMP alone show the mean ± S.E.M. of at least three filters. The data presented for the combination of PMA and 8Br-cAMP are representative of two experiments. &Significantly lower than the value obtained with PMA ($P < 0.05$). *Significantly lower than the value without chronic phorbol ester treatment ($P < 0.05$).

and in the combination experiment only the stimulation by 8Br-cAMP was left.

The V_2 receptor agonist dDAVP (10 nM; basolateral side) evoked a similar increase in the amount of [^{3}H]PtdEth as ATP and 8Br-cAMP (Fig. 3B). In combination with ATP, the amount of [^{3}H]PtdEth was further increased by a factor 2 as compared to the effect of each stimulus alone. Chronic PMA treatment did not affect the stimulatory action of dDAVP, while in the combination it decreased the amount of [^{3}H]PtdEth practically to the value ob-

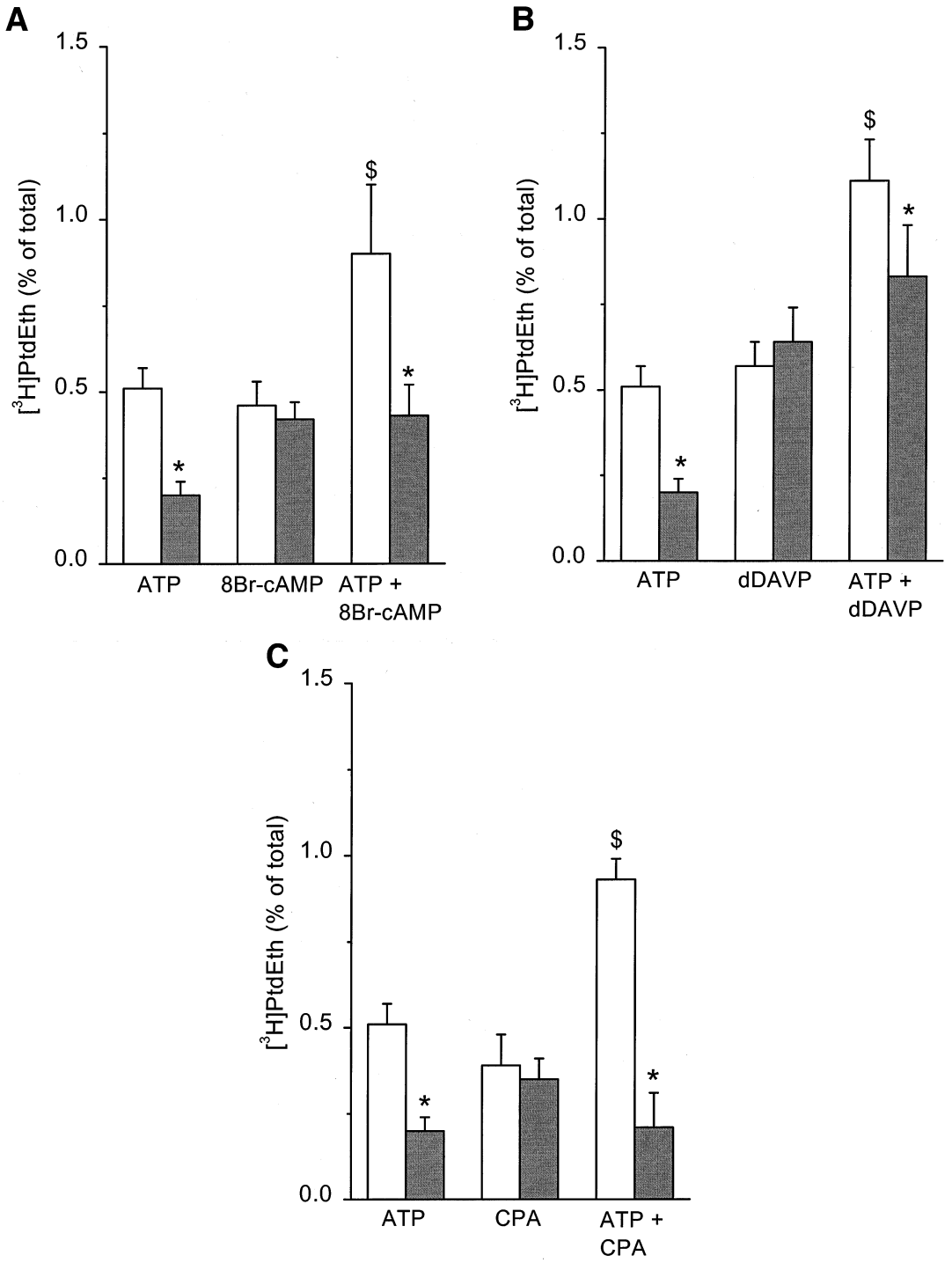

Fig. 3. Chronic phorbol ester treatment does not affect 8Br-cAMP, dDAVP or CPA activation of PLD but markedly reduces the effect of ATP. Chronic phorbol ester treatment (closed bars) and PLD activity measurements were performed as described in the legend of Fig. 2. Additions were: (A) ATP (0.1 mM; both sides) and/or 8Br-cAMP (0.1 mM; both sides), (B) ATP (0.1 mM; both sides) and/or dDAVP (10 nM; basolateral side), and (C) ATP (0.1 mM; both sides) and/or CPA (10 μM; apical side). Of note, the values for 8Br-cAMP are also shown in Fig. 2. For each filter, the amount of [^{3}H]PtdEth is expressed as the percentage of total labelled lipid that was extracted. The data presented show the mean ± S.E.M. of at least three filters. $^{\$}$Significantly higher than the value obtained with either stimulant alone ($P < 0.05$). *Significantly lower than the value without chronic phorbol ester treatment ($P < 0.05$).

tained with dDAVP alone. Previous work showed that dDAVP evokes a marked increase in cAMP that is virtually abolished by the potent adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine (DDA) [4]. To test whether this increase in cAMP is responsible for dDAVP-induced PLD activation, monolayers were stimulated with dDAVP in the presence of DDA (100 μM, both sides) added 15 min prior to stimulation. DDA did not significantly affect the dDAVP-induced increase in [^{3}H]PtdEth ($0.74 \pm 0.11\%$, $n = 3$ and $0.72 \pm 0.03\%$, $n = 4$ in control and DDA-treated monolayers, respectively).

The adenosine A_1 receptor agonist CPA (10 μM; apical side) increased the amount of [^{3}H]PtdEth to the same extent as 8Br-cAMP, ATP or dDAVP (Fig. 3C). When added in combination with ATP a 1.8-fold increase in [^{3}H]PtdEth formation was observed as compared to the value obtained with ATP alone. Chronic PMA treatment did not affect the stimulatory action of CPA and reduced the effect of the combination to the value obtained with CPA alone. CPA-induced PLD activation was not affected in monolayers pretreated with pertussis toxin (170 ng/ml) for 24 h, indicating that CPA does not act via G_i (data not shown).

Table 1 shows that the effects of dDAVP and 8Br-cAMP were not additive, whereas a 1.5-fold increase in [^{3}H]PtdEth formation was observed with the combination of CPA and 8Br-cAMP as compared to the effect of 8Br-cAMP alone. Finally, no additive effect was observed with the combination of CPA and dDAVP. In none of these cases did the values obtained with vehicle-treated monolayers differ from those obtained following chronic PMA treatment.

Table 1
Stimulatory hormones for Ca^{2+} reabsorption and 8Br-cAMP do not increase PLD activity in an additive manner

Stimulant	[^{3}H]PtdEth (% of total)	
	No treatment	Chronic PMA treatment
8Br-cAMP	0.46 ± 0.07	0.42 ± 0.05
dDAVP	0.57 ± 0.07	0.64 ± 0.10
CPA	0.39 ± 0.09	0.35 ± 0.06
8Br-cAMP+dDAVP	0.76 ± 0.07	0.77 ± 0.04
8Br-cAMP+CPA	0.68 ± 0.11	0.51 ± 0.09
dDAVP+CPA	0.56 ± 0.09	0.46 ± 0.07

Chronic phorbol ester treatment and PLD activity measurements were performed as described in the legend of Fig. 2. Additions were: 8Br-cAMP (0.1 mM; both sides), dDAVP (10 nM; basolateral side) and CPA (10 μM; apical side). For each filter, the amount of [^{3}H]PtdEth is expressed as the percentage of total labelled lipid that was extracted. Of note, the values for 8Br-cAMP, dDAVP and CPA alone are also shown in Figs. 2 and 3. The data presented show the mean ± S.E.M. of at least three filters. In none of the cases was the value obtained with the combination significantly higher than that obtained with the most active stimulant alone.

To investigate the role of PKC in more detail monolayers were stimulated in the presence of the selective PKC inhibitor chelerythrine (10 μM, both sides) added 15 min prior to stimulation. In the presence of chelerythrine, basal [^{3}H]PtdEth formation amounted to $0.40 \pm 0.06\%$ ($n = 5$) of total [^{3}H]myristic acid-labelled lipid. PMA-induced [^{3}H]PtdEth formation was reduced by 60% in monolayers treated with chelerythrine (Fig. 4A). Similarly, this inhibitor virtually abolished the stimulatory action of 8Br-cAMP. ATP-induced [^{3}H]PtdEth formation was not affected by chelerythrine, while the stimulatory actions of dDAVP and CPA were decreased by 90% and 60%, respectively (Fig. 4B).

4. Discussion

This study demonstrates that Ca^{2+}-transporting cells of rabbit CNT and CCD contain a regulated PLD activity which is, however, not involved in regulated Ca^{2+} reabsorption. This conclusion is based on the finding that both stimulatory and inhibitory hormones for Ca^{2+} transport promote the transphosphatidylation reaction in primary cultures of rabbit CNT and CCD cells. This observation also disfavours the idea that selective activation of PtdCho-specific PLD by either stimulatory or inhibitory hormones underlies their differential activation of phorbol ester-insensitive and -sensitive PKC isoforms.

Thus far, two mammalian isoforms of PLD, PLD1 and PLD2, have been cloned from a wide variety of species [27]. In addition, two alternatively spliced forms of PLD1 (PLD1a and PLD1b), and three alternatively spliced forms of PLD2 (PLD2a, PLD2b and PLD2c) have recently been identified [28,29]. At present, information on the expression levels, cellular localisation and function of PLD1 and PLD2 proteins is limited. Using an antibody raised against N- and C-terminal sequences of human PLD1 [24] we now show that rabbit cortical collecting system cells

contain substantial amounts of this PLD isoform. RT-PCR on total RNA suggests that it concerns the 1b splice variant of PLD1. In addition, the cells gave a clear signal with specific primers for PLD2. At present, however, we do not know to what extent this latter isoform is expressed at the protein level. Thus far, Northern blot analysis has revealed the presence of PLD1 as well as PLD2 transcripts in human and rat kidney (listed in [30]).

Chronic phorbol ester treatment (120 h) did not affect PLD activation by the stimulatory hormones CPA and dDAVP, while it markedly reduced PLD activation by the inhibitory hormone ATP. These findings suggest that both the stimulatory and inhibitory pathways for Ca^{2+} reabsorption lead to PLD activation (for a schematic model, see Fig. 5). The effectiveness of chronic phorbol ester treatment was demonstrated by abolition of PMA-induced PLD activity. Chronic phorbol ester treatment has been used in many cell systems to demonstrate the involvement of phorbol ester-sensitive PKC isoforms in hormone-stimulated PLD activity [26,31,32]. In other cell models, however, such treatment did not affect hormone-stimulated PLD activity [33–35]. In this context, rabbit cortical collecting system cells represent a unique system in that chronic phorbol ester treatment inhibits its PLD activation by one group of hormones (represented by ATP), while it does not affect PLD activation by another group of hormones (represented by dDAVP and CPA).

The present finding that hormones that stimulate Ca^{2+} reabsorption increase PLD activity is in line with the observation that PTH(1–84) stimulated the transphosphatidylation reaction in immortalised mouse distal convoluted tubule cells [18]. However, our finding that the inhibitory nucleotide ATP, which in itself does not change basal Ca^{2+} reabsorption [9], markedly increases PLD activity disfavours the idea put forward by these authors that PLD may represent the non-PtdIns-PLC pathway generating

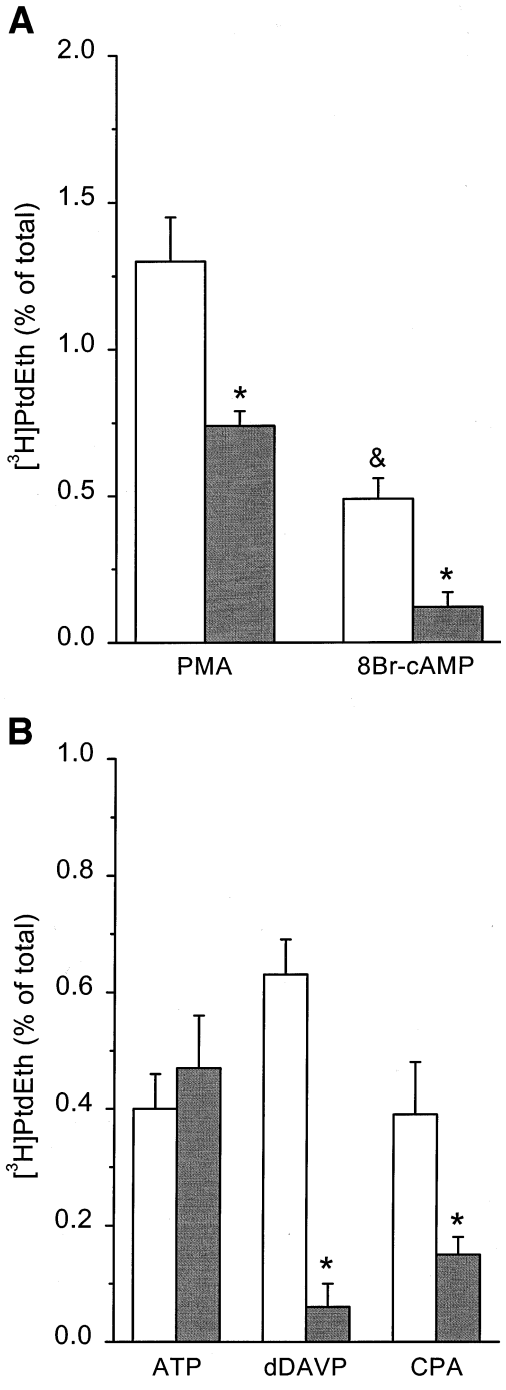

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Fig. 4. Chelerythrine does not affect the ATP-induced increase in PLD activity but markedly reduces the effects of 8Br-cAMP, dDAVP, CPA and PMA. PLD activity measurements were performed as described in the legend of Fig. 2. Monolayers were stimulated in the presence of chelerythrine (10 μM, both sides) added 15 min prior to stimulation (closed bars). Additions were: (A) PMA (0.1 μM; both sides) and 8Br-cAMP (0.1 mM; both sides), and (B) ATP (0.1 mM; both sides), dDAVP (10 nM; basolateral side) and CPA (10 μM; apical side). For each filter, the amount of [^{3}H]PtdEth is expressed as the percentage of total labelled lipid that was extracted. The data presented show the mean ± S.E.M. of at least three filters. &Significantly lower than the value obtained with PMA ($P < 0.05$). *Significantly lower than the value without chronic phorbol ester treatment ($P < 0.05$).

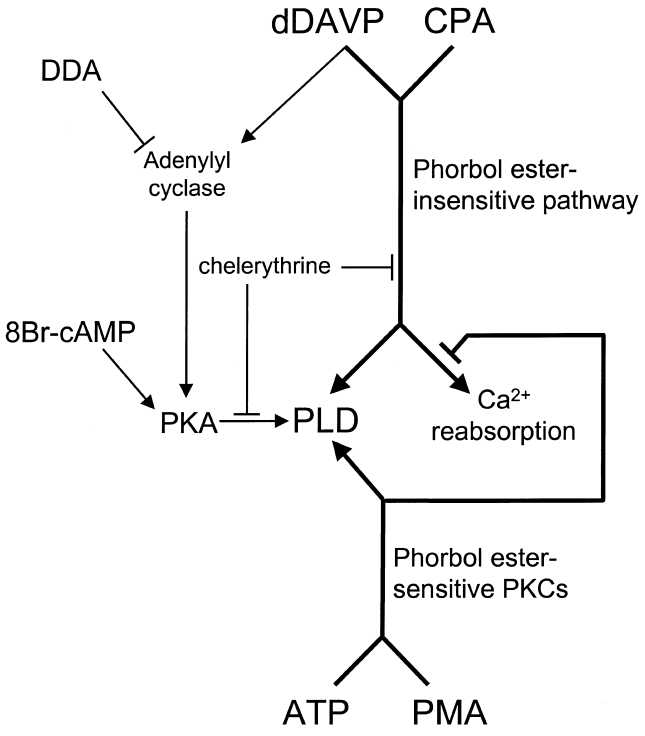

Fig. 5. Schematic model of the hormonal regulation of PLD activity in rabbit CNT and CCD cells. For explanation see text.

the diacylglycerols for PKC-dependent Ca^{2+} reabsorption.

In contrast to chronic phorbol ester treatment, chelerythrine markedly inhibited PLD activation by the stimulatory hormones CPA and dDAVP. This finding is in agreement with the previous observation that chelerythrine effectively inhibits the stimulatory effect of these hormones on Ca^{2+} reabsorption [4], and stresses the idea that the same phorbol ester-insensitive, chelerythrine-inhibitable pathway that leads to increased Ca^{2+} reabsorption also leads to PLD activation (see also Fig. 5). Chelerythrine did not affect PLD activation by the inhibitory hormone ATP. Nor did chelerythrine abolish the increase in PLD activity evoked by PMA. These findings may suggest that the phorbol ester-sensitive PKC pathway used by ATP to activate PLD and inhibit hormone-stimulated Ca^{2+} reabsorption is less sensitive to chelerythrine than the phorbol ester-insensitive pathway used by the stimulatory hormones.

In addition to PMA, 8Br-cAMP markedly increased PLD activity in these primary cultures. This suggests the presence of a cAMP-dependent pathway leading to PLD activation. Recently, the existence of such a pathway has been reported in FRTL-5 thyroid cells [36]. Chronic phorbol ester treatment did not affect 8Br-cAMP stimulation of PLD activity, indicating that 8Br-cAMP, similar to dDAVP and CPA but in contrast to ATP and PMA, does not require the presence of phorbol ester-sensitive PKC isoforms for activation of PLD. Similar to dDAVP and CPA stimulation of PLD activity, 8Br-cAMP stimulation of PLD activity was virtually abolished by chelerythrine. Importantly, however, the dDAVP-induced increase in PLD activity was not reduced by the potent adenylyl cyclase inhibitor DDA, indicating that dDAVP does not act via the cAMP-dependent pathway. Similarly, the involvement of the cAMP-dependent pathway in the mechanism of action of CPA is excluded by the fact that CPA does not increase cAMP in these monolayers [7]. These findings show that dDAVP and CPA do not act through PKA to stimulate PLD activity.

When the inhibitory nucleotide ATP was added in combination with either 8Br-cAMP, dDAVP or CPA, the increase in PLD activity was higher than that observed with each of the stimulants alone. These observations are in agreement with the idea that ATP and the stimulatory hormones activate PLD via two distinct pathways. Conversely, none of the combinations of the stimulatory hormones gave an additive effect. This latter observation suggests that the pathway used by the stimulatory hormones is already maximally activated by each of the hormones. The observation that addition of 8Br-cAMP did not lead to a further increase of PMA-stimulated PLD activity then suggests that the phorbol ester-sensitive PKC pathway, in contrast to the phorbol ester-insensitive pathway, can fully activate PLD.

It is unknown at which of the steps involved in transepithelial Ca^{2+} transport, i.e. apical Ca^{2+} influx, cytosolic diffusion of Ca^{2+} bound to calbindin or basolateral Ca^{2+} extrusion, the regulatory hormones act. However, voltage-clamp studies with *Xenopus laevis* oocytes heterologously expressing the recently cloned ECaC provide evidence for a strong inhibition of Ca^{2+} influx by intracellular Ca^{2+}, suggesting that ECaC is a major site for regulation of Ca^{2+} reabsorption [37]. Moreover, elucidation of the primary structure of ECaC revealed the presence of seven potential PKC phosphorylation sites [3], and future

studies will decide whether or not these sites are involved in the hormonal regulation of Ca^{2+} reabsorption.

In conclusion, the present observation that both hormones that stimulate and those that inhibit Ca^{2+} reabsorption readily increase PLD activity suggests that PLD activation does not play a role in the mechanism by which these hormones exert their regulatory action. The physiological relevance of PLD activation in CNT and CCD cells is at present unclear and awaits further investigation.

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References

[1] R.J. Bindels, J. Exp. Biol. 184 (1993) 89–104.
[2] P.A. Friedman, F.A. Gesek, Physiol. Rev. 75 (1995) 429–471.
[3] J.G.J. Hoenderop, A.W.C.M. van der Kemp, A. Hartog, S.F. van de Graaf, C.H. van Os, P.H.G.M. Willems, R.J.M. Bindels, J. Biol. Chem. 274 (1999) 8375–8378.
[4] J.G.J. Hoenderop, J.J.H.H.M. De Pont, R.J.M. Bindels, P.G.H.M. Willems, Kidney Int. 55 (1999) 225–233.
[5] J. Van Baal, M.D. de Jong, F.J. Zijlstra, P.H.G.M. Willems, R.J.M. Bindels, J. Physiol. 497 (1996) 229–239.
[6] J. Van Baal, G. Raber, J. de Slegte, R. Pieters, R.J.M. Bindels, P.H.G.M. Willems, Pflügers Arch. 433 (1996) 109–115.
[7] J.G.J. Hoenderop, A. Hartog, P.H.G.M. Willems, R.J.M. Bindels, Am. J. Physiol. 274 (1998) F736–F743.
[8] J.G.J. Hoenderop, A.B. Vaandrager, L. Dijkink, A. Smolenski, S. Gambaryan, S.M. Lohmann, H.R. de Jonge, P.H.G.M. Willems, R.J.M. Bindels, Proc. Natl. Acad. Sci. USA 96 (1999) 6084–6089.
[9] J. Van Baal, J.G.J. Hoenderop, M. Groenendijk, C.H. van Os, R.J.M. Bindels, P.H.G.M. Willems, Am. J. Physiol. 277 (1999) F899–F906.
[10] H.P.G. Koster, A. Hartog, C.H. van Os, R.J.M. Bindels, Am. J. Physiol. 270 (1996) F53–F60.
[11] R.J.M. Bindels, J.A. Dempster, P.L.M. Ramakers, P.H.G.M. Willems, C.H. van Os, Kidney Int. 43 (1993) 295–300.
[12] P. Poujeol, M. Bidet, M. Tauc, Kidney Int. 48 (1995) 1102–1110.
[13] P.A. Friedman, B.A. Coutermarsh, S.M. Kennedy, F.A. Gesek, Endocrinology 137 (1996) 13–20.
[14] V. Natarajan, W.M. Scribner, S. Vepa, Chem. Phys. Lipids 80 (1996) 103–116.
[15] J.H. Exton, Biochim. Biophys. Acta 1212 (1994) 26–42.
[16] Y. Nishizuka, Science 258 (1992) 607–614.
[17] M.N. Hodgkin, T.R. Pettitt, A. Martin, R.H. Michell, A.J. Pemberton, M.J.O. Wakelam, Trends Biosci. 23 (1998) 200–204.
[18] P.A. Friedman, F.A. Gesek, P. Morley, J.F. Whitfield, G.E. Willick, Endocrinology 140 (1999) 301–309.
[19] G. Liu, L. Kleine, R. Nasrallah, R.L. Hébert, Am. J. Physiol. 276 (1999) F589–F598.
[20] R.M. Dawson, Biochem. J. 102 (1967) 205–210.
[21] R.J.M. Bindels, A. Hartog, J. Timmermans, C.H. Van Os, Am. J. Physiol. 261 (1991) F799–F807.
[22] K. Katayama, T. Kodaki, Y. Nagamachi, S. Yamashita, Biochem. J. 329 (1998) 647–652.
[23] T. Kodaki, S. Yamashita, J. Biol. Chem. 272 (1997) 11408–11413.
[24] V. Müller-Wieprecht, C. Riebeling, C. Alexander, F.R. Scholz, A. Hoer, T. Wieder, C.E. Orfanos, C.C. Geilen, FEBS Lett. 425 (1998) 199–203.
[25] R.R. Bosch, A.M.P. Patel, S.E. Van Emst-de Vries, R.L.L. Smeets, J.J.H.H.M. De Pont, P.H.G.M. Willems, Eur. J. Pharmacol. 346 (1998) 345–351.
[26] R.R. Bosch, R.L.L. Smeets, F. Sleutels, A.M.P. Patel, S.E. Van Emst-de Vries, J.J.H.H.M. De Pont, P.H.G.M. Willems, Biochem. J. 337 (1999) 263–268.
[27] J.H. Exton, Biochim. Biophys. Acta 1439 (1999) 121–133.
[28] S.M. Hammond, J.M. Jenco, S. Nakashima, K. Cadwallader, Q. Gu, S. Cook, Y. Nozawa, G.D. Prestwich, M.A. Frohman, A.J. Morris, J. Biol. Chem. 272 (1997) 3860–3868.
[29] I. Lopez, R.S. Arnold, J.D. Lambeth, J. Biol. Chem. 273 (1998) 12846–12852.
[30] K.E. Meier, T.C. Gibbs, S.M. Knoepp, K.M. Ella, Biochim. Biophys. Acta 1439 (1999) 199–213.
[31] M.A. Balboa, B.L. Firestein, C. Godson, K.S. Bell, P.A. Insel, J. Biol. Chem. 269 (1994) 10511–10516.
[32] S. Gupta, A. Gomez-Munoz, W.C. Matowe, D.N. Brindley, J. Ginsberg, Endocrinology 136 (1995) 3794–3799.
[33] M. Schmidt, S.M. Huwe, B. Fasselt, D. Homann, U. Rumenapp, J. Sandmann, K.H. Jakobs, Eur. J. Biochem. 225 (1994) 667–675.
[34] M.A. Balboa, P.A. Insel, Mol. Pharmacol. 53 (1998) 221–227.
[35] J. Sandmann, R.J. Wurtman, J. Neurochem. 56 (1991) 1312–1319.
[36] J. Ginsberg, S. Gupta, W.C. Matowe, L. Kline, D.N. Brindley, Endocrinology 138 (1997) 3645–3651.
[37] J.G.J. Hoenderop, A.W.C.M. van der Kemp, A. Hartog, C.H. van Os, P.H.G.M. Willems, R.J.M. Bindels, Biochem. Biophys. Res. Commun. 261 (1999) 488–492.